# TOPICAL APPLICATION OF ACYCLOVIR-LOADED MICROPARTICLES: QUANTIFICATION OF THE DRUG IN PORCINE SKIN LAYERS.

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**ABSTRACT**

The goal of this work was to increase the amount of acyclovir (ACV) in the basal epidermis, site of Herpes virus simplex infections, using microparticles as carriers. Poly(DL-lactic-co-glycolic acid) (PLGA)-microparticles loaded with acyclovir were prepared using a solvent evaporation technique. ACV distribution into porcine skin after topical application of microparticles, during 6, 24 and 88 h, was determined by horizontal slicing of the skin. ACV suspension served for comparison. The results showed that, at 6 and 24 h, the quantity of the drug in the basal epidermis, with the microparticles, is similar to that obtained with the acyclovir suspension. However, after 88 h, the acyclovir reservoir in the basal epidermis was higher with microparticles compared with the control suspension. This fact could be explained by the controlled drug released produced by the vector in the basal epidermis. Besides, at 88 h the amount of acyclovir detected in the receptor chamber of the diffusion cells was much lower with the microparticles than with the suspension. This kind of carriers can improve acyclovir topical therapy since they increased drug retention in the basal epidermis and consequently increased the time intervals between doses.

**KEYWORDS**

PLGA-microparticles, Acyclovir, Drug skin distribution, Porcine skin.

## 1. INTRODUCTION

Acyclovir (ACV), a synthetic analogue of 2'-deoxiguanosine, is one of the most effective and selective agents against viruses of the herpes group. ACV is active against herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2), varicella zoster virus, and in a less extent against Epstein-Barr virus and cytomegalovirus. The mechanism of action of this drug has been extensively studied, and its antiviral activity has been shown to result from the inhibition of herpesvirus DNA replication [1].

ACV was effective against cutaneous infections due to HSV-1, whose target site is the basal epidermis. However, it has been suggested that ACV topical therapy has a low efficacy, due to the lack of penetration of an enough amount of drug to the target site [2]. In this way, Parry et al. [3] found a good relationship between the free drug concentration at the basal epidermis and the *in vivo* antiviral efficacy for a variety of ACV topical formulations. Consequently, the quantification of ACV within the different strata of the skin will be essential to determinate its effectiveness.

The literature reports have documented different methodologies for quantifying drug amounts within the skin e.g. skin extraction measurements [4], horizontal stripping and sectioning [5], quantitative autoradiography and spectroscopic methods [6-7]. Horizontal sectioning on a freeze microtome provides information on drug localisation and allows to determinate the penetrant concentration-depth profile. For that reason, this method has been employed by several authors [3,8] in order to obtain the ACV concentration at different skin depths.

The development of an effective ACV topical preparation prompted researchers to try several approaches. In some studies, superior antiviral activity was demonstrated using differents vehicles [9], percutaneous absorption enhancers [10] and iontophoresis [8]. Another possible strategy to achive site-specific drug delivery [11] would be the use of

particulate drug carriers (microparticles and nanoparticles). These carriers possess some advantages for the topical application, since the sustained release is important to supply the skin with the drug over a prolonged period of time. Indeed, Boutounne et al. [12] reported that chlorhexidine nanoparticles allowed to improve the drug permanence into the skin and to decrease the chrlorhexidine transdermal delivery. Also, Jenning et al. [13] showed that vitamin A loaded solid lipid nanoparticles delivered effectively the drug to the upper skin layers, but in the deeper skin strata the drug levels appeared not to increase.

The objective of this work was to obtain a high quantity of ACV in the basal epidermis, where viral lesions are usually located, with the use of ACV loaded microparticles. For this purpose, the amount of ACV accumulated in the different strata of porcine skin was determinated and compared with a control suspension. Besides, the flux through the skin into the systemic circulation was evaluated with these formulations. This study also involves the preparation of PLGA microparticles containing ACV, their characterisation and their *in vitro* evaluation.

## 2. MATERIALS AND METHODS

### 2.1. Materials

Acyclovir was a gift from Glaxo-Wellcome (Madrid, Spain). Poly (D,L-lactid-co-glycolid acid) (PLGA) Resomer® RG 502 H, 12000 daltons MW, have been purchased from Boehringer Ingelheim (Ingelheim, Germany). Polyvinyl alcohol (PVA) MW 115000 was supplied by BDH (Poole, England) and dichloromethane was provided by Prolabo (Fontenay, France). All other chemicals were obtained from Sigma (St. Louis, USA) and Merck (Darmstadt, Germany) and they were of HPLC analytical grade.

## 2.2. Preparation of microparticles

ACV-containing microparticles were prepared using a solvent evaporation technique. 100 mg of ACV were dispersed in 16% (w/v) polymer solution in dichloromethane. The resulting dispersion was added to 30 mL of a 0.5% PVA solution and homogenised using an ultraturrax® (Euro Turrax T20b IKA Labortechnik, Staufen, Germany) for 1 minute. This mixture was stirred at 25ºC for at least 3 h until complete solvent evaporation. Microparticles were collected by centrifugation (3000 rpm for 10 min, Biofuge stratus Heraeus Instruments, Hanau, Germany), washed 3 times with distilled water, freeze-dried for 48 h (Virtis Genesis 12 EL, Gardines, NY) and stored at 4ºC.

An ACV suspension in water containing 15 mg of drug was used as a control. The particle size and the polydispersity of the suspension were measured by laser light diffraction (Mastersizer® S, Malvern Instruments, UK).

## 2.3. Microparticle characterisation

*2.3.1. Morphology and size of the microparticles*

For morphological examinations, microparticles were placed on sample holders, 16 nm gold-coated (Emitech K550, England) and then viewed with a Scanning Electron Microscopy (SEM; Scanning digital electron microscope DSM-940A, Zeiss, Germany). Microspheres diameter and size distribution were measured by laser light diffraction (Mastersizer® S, Malvern Instruments, UK). The average particle size was expressed as the volume mean diameter in micrometers.

*2.3.2. Determination of ACV content in the microparticles*

The ACV content in the PLGA microspheres was determinated by using a UV-spectrophotometer (Diode-array HP 8452 AX, Waldbronn, Germany) at 252 nm**.** The ACV loaded microspheres were dissolved in 2 mL of dichloromethane, and the drug

was extracted twice with 6 mL of NaOH $10^{-4}$ M. The entrapment efficiency of ACV was calculated as the ratio of actually measured to theoretical (nominal) drug content in microspheres.

*2.3.3. In vitro ACV release studies*

*In vitro* drug release profiles were obtained by incubating the microparticles (approx. 5 mg accurately weighed) in a 1.5 mL of phosphate-buffered saline (PBS) (pH 7.4) containing 0.02% sodium azide as a bacteriostatic agent. Incubation took place in rotating vials at 37ºC. At predetermined time intervals (1, 6 h and 1, 2, 4,7 days), the samples were centrifuged at 17000 rpm for 15 min, and ACV concentration in the supernatant was quantified by UV spectrophotometer at 252 nm.

**2.4. Skin permeation experiments**

Porcine ears were obtained from the local slaughterhouse and after cleaning them under cold running water, the outer region of the ear was cut. The whole skin was dermatomed (AESCULAP®, Tuttlingen, Germany) to 1.2 mm and immediately frozen at –20ºC. Skin was allowed to hydrate for 1 h before being mounted on the Franz type diffusion cells, with an available diffusion area of 1.76 $cm^2$, (FDC-400, Grown Glass Company, Somerville, NY), with the stratum corneum facing the donor compartment.

In this study, 1 mL of microparticles containing ACV (15 mg) or the drug suspension (15 mg) were placed, on the skin surface, in the donor chamber. The receptor chamber contained 11 mL of phosphate buffer solution (pH 7.4) consisted of 1.787 g of $KH_2PO_4$ 1/15 M and 9.531 g of $Na_2HPO_4$ 1/15 M in 1 L with a ionic strength of 0.266 M. At given time intervals, 400 μL aliquots were collected and replaced with the same volume of fresh buffer. The receptor medium was maintained at 37±1ºC and stirred at 600 rpm using magnetic stirring bars.

The samples were analysed by high-performance liquid chromatography (HPLC Hewlett-Packard 1100) with ultraviolet detection (λ=252 nm). The column used was a reversed-phase 250X4 mm $C_8$ LiChrospher Select B (5 μm) provided by Merck. The mobile phase consisted of acetonitrile/ammonium acetate (1:99). The flow-rate was 1.0 mL $min^{-1}$ and the temperature was 20ºC. The HPLC method for ACV quantification either in the skin or in the receptor compartment has been previously validated. The detector response was found to be linear in the concentration range 0.05 to 10 μg/mL (r>0.999). Accuracy and precision values were always below 5% and the limit of quantification of the method was 0.009 μg/mL.

**2.5. Horizontal skin sectioning and drug extraction**

After permeation experiments, the skin was removed (1.76 $cm^2$) and rinsed with distilled water. This procedure was repeated three times. Then, the skin was frozen in liquid nitrogen and cut with a freeze microtome (2800 Frigocut E, Reichert-Jung, Germany) in order to get horizontal slices of 30 μm. ACV was extracted from the slices with 200 μl of distilled water at 60ºC ± 5ºC for 15 min. After cooling, perchloric acid was added to precipitate proteins and the mixture was centrifuged at 8000 rpm (Biofuge stratus Heraeus Instruments, Germany). The extract was then analysed by HPLC. For the calibration procedure, blank samples of slices of skin were spiked with three different known amount of ACV solution, and after 3 h of contact, were extracted as previously described. The extraction recovery was measured comparing the amount of ACV added and extracted. The skin slices were from the different animals and from different depths. Satisfactory recoveries were obtained from all samples tested (>95%).

ACV concentration was expressed as μg of ACV/$cm^3$ of skin.

**2.6. Statistics**

All data are presented as arithmetic mean values ± standard error (S.E.). Significant differences were analysed using Shapiro-Wilk, F-, and Student's t-test, $P \leq 0.05$ was considered significant [13].

## 3. RESULTS AND DISCUSION

### 3.1. Microparticles characterisation

Microparticles had unimodal distribution with diameters between 1 and 10 μm and a volume mean diameter of 4.7 ± 0.28 μm. SEM micrographs of the particles showed spherical and well individualized microspheres (Fig. 1). The encapsulation efficiency was 50±5% (62.5 μg ACV/mg polymer).

The release profiles of ACV from PLGA microparticles were carried out in PBS pH 7.4, drug release within the first hour (also called burst release) was 80% of the actual loading, corresponding to 50 μg ACV/ mg polymer. After the burst, an additional 20% of the drug was released within 7 days (Fig. 2). The amount of ACV remaining in the microparticles after 7 days, (after dissolving the particles in dichloromethane and extracting the ACV with 6 mL of NaOH $10^{-4}$ M), was 3.07 ± 1.03 mg. These results suggest that most of ACV is located at the particles surface, although a part of ACV is entrapped within the particles. For topical application both features are of interest, since burst release can be useful to improve the initial penetration of the drug and sustained release becomes important to supply the skin with ACV over a prolonged period of time.

Recently, several authors proposed microparticles loaded with ACV for ophthalmic administration. Conti et al. [14] achieved good encapsulation efficiencies by spray-drying technique, although the mean particle size (7 μm) was bigger than the one presented in this work. Genta et al. [15] have also prepared acyclovir-loaded chitosan

microspheres by a W/O/W technique with similar size to ours, although the encapsulation efficiency was much lower (between 14.4 and 28.71%).

### 3.2. Skin distribution of ACV loaded microparticles

In a previous work [16-17] we have shown that PLGA microparticles can penetrate through stratum corneum and reached the basal epidermis, the target site of the HSV-1 infection.

ACV distribution into porcine skin, after topical application of microparticles during 6, 24 and 88 h, was determined by horizontal slicing of the skin. ACV suspension in water served for comparison. The particle size of the drug in the suspension used was 8.65 ± 0.24 μm with an unimodal distribution. In this case, the solubility of ACV at 37ºC was 2.35 mg/mL.

Porcine skin was chosen because it is structurally the most similar to human skin [18-19] and it is well suited for representing the human skin permeability [20]. Jenning et al. [13] reported, using the light microscopy, that in the porcine skin, the upper 100 μm represent mainly the stratum corneum and upper layers of viable epidermis and between 100-200 μm consists basically of viable epidermis and the dermis is located from 200 to 500 μm. In the same way, Parry et al. [3] assumed that human skin has a thickness of 40 μm for hydrated stratum corneum, 115 μm for the epidermis and 460 μm for the dermis. According to these data and to the skin morphology of the different slices, we assumed that basal epidermis (site of herpes virus lesions), it is located between 120-150 μm.

Fig. 3a showed the quantity of ACV detected, in the different strata of skin, 6 h after topical application of microparticles (15 mg of drug) and the control suspension (15 mg). The amount of ACV found, until 180 μm of depth, was slightly higher with microparticles than with the control suspension. However, from 180 to 600 μm, there were no significant differences between the two formulations. The higher concentration

in the first layers of the skin, after microparticles application, could be explained by the occlusive effect, since microparticles produced a film, on the skin surface, which reduces the transepidermal water loss and favours drug penetration into the skin. According to these data, several authors [13,21] have observed the same feature using solid lipid nanoparticles.

Fig. 3b showed the distribution profile of ACV in different skin layers at 24 h. The profiles obtained were similar to that reported at 6 h, but at 24 h the amount of the drug found in all depths was higher. At this time, no significant differences were found between the microparticles and the suspension.

During the first 24 h of the experiment, the ACV concentration found into the skin, after microparticles application, is mainly due to the free ACV coming from the initial burst release. In order to evaluate the sustained release of these forms experiments at 88 h were carried out, since at this time, ACV loaded microparticles started to release the encapsulated drug. Besides, the integrity of the membrane barrier can not be confirmed for a longer period of time. To guarantee this integrity, the skin was observed by light microscopy before and after the permeation experiments, and no skin structural alterations were found. At 88 h, different profiles were obtained with microparticles and control suspension (Fig. 3c). Indeed, with microparticles, the amount of ACV accumulated in the different strata of skin showed a similar profile to that obtained at 6 or 24 h, although the concentration of the drug in different layers was higher. However, with the ACV suspension, the distribution profile at 88 h was totally different, in this case the accumulation of ACV in the skin increased until 300 μm, and from this depth to 600 μm the profiles resulted practically constant versus depth. At the basal epidermis, (120-150 μm) targed site of herpes virus, the quantity of ACV found with microparticles was higher that with the suspension (significant differences, $P \leq 0.05$,

were found) and this difference pointed out the relevance of this carrier system for ACV topical delivery.

Several studies [1] have shown that at concentration of about 0.01 to 0.7 μg/mL, ACV can be useful for a 50% inhibition of the viral cytopathic effect ($ID_{50}$). According to the results, tissue concentrations obtained after microparticles application were higher than the $ID_{50}$ reported.

The ACV concentration in the receptor side of the diffusion cells, from microparticles and control suspension, are shown in the Fig. 4. In both experiments, 15 mg ACV were applied to the skin. During the first 24 h, there were no differences in the amount of ACV in the receptor medium. Nevertheless, 88 h after topical application of both formulations, a 3.45 times higher drug concentration was measured for the control suspension compared to microparticles. These data were in agreement with the amount of ACV accumulated in the different strata of porcine skin, since at 88 h, the quantity of the drug found in deep strata (600 μm) was much higher with the control suspension than with microparticles (Fig. 3c). Consequently, this work showed that the use of ACV loaded microparticles increases drug retention in the basal epidermis and decreases the drug permeation through the skin.

## 4. CONCLUSIONS

ACV loaded PLGA microparticles, prepared using a solvent evaporation technique, yields uniform size microspheres and a good encapsulation efficiency. The distribution profile of ACV in different skin layers, after topical application of microparticles, at 6 and 24 h are similar to that obtained with the control suspension. However, at 88 h the microparticles provided higher concentration of ACV in the basal epidermis than

control suspension. This result is in agreement with the greater ACV concentration found in the receptor compartment with the control suspension at 88 h.

Consequently, microparticles represent a good delivery system to retard the release rate of ACV into the skin. Indeed, they could improve topical therapy by increasing the time intervals between doses.

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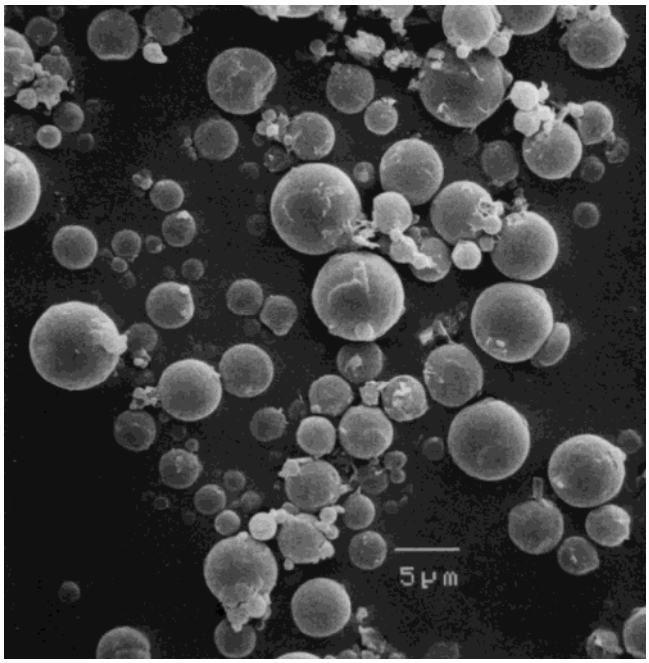



**Fig. 1.**
**Figure 1.** Scanning electron micrograph of ACV loaded microparticles (bar = 5 μm).

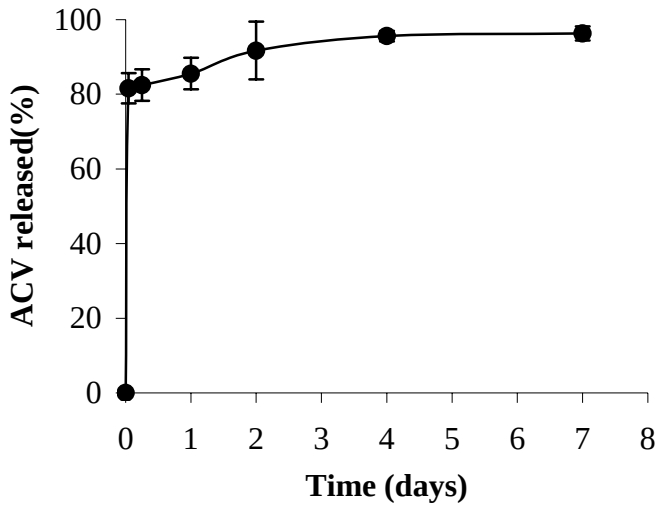
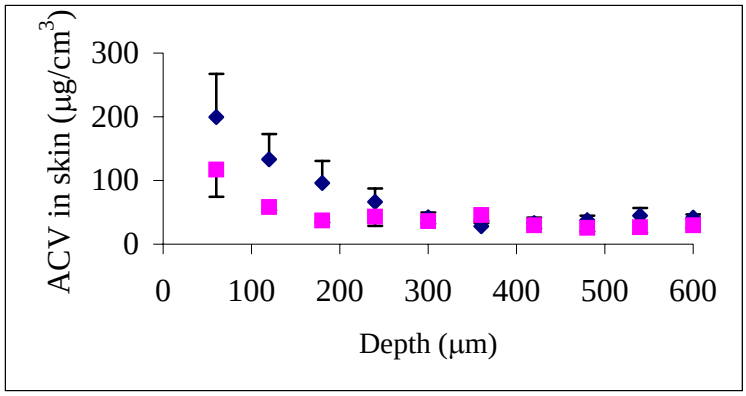
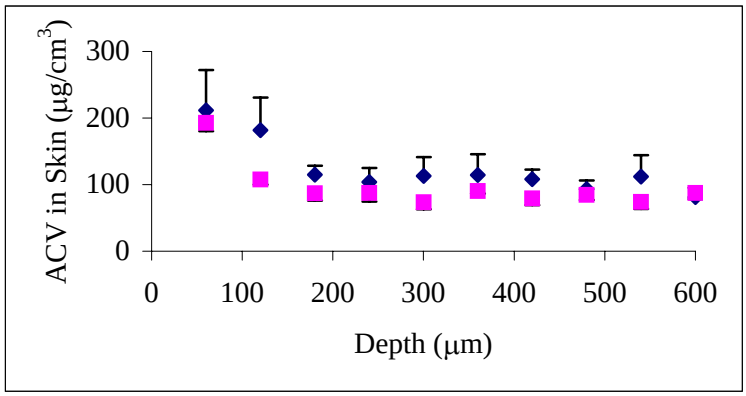



**Fig. 2.**

**Figure 2.** *In vitro* release of ACV from PLGA microparticles in PBS at 37ºC (mean±S.D., n=3).




**(a)**

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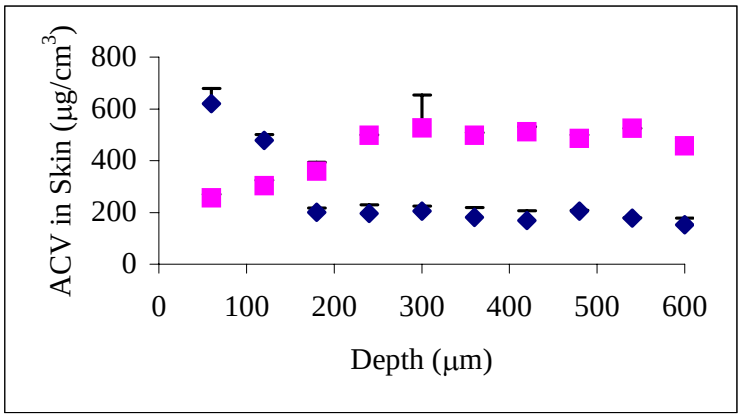


**(c)**

**Figure 3.** Distribution of ACV, in porcine skin layers, following topical administration of microparticles (u) and control suspension (n) as a function of time:(a) 6 h (b) 24 h (c) 88 h. Each point represents the mean value ± S.E. (*n* = 3).

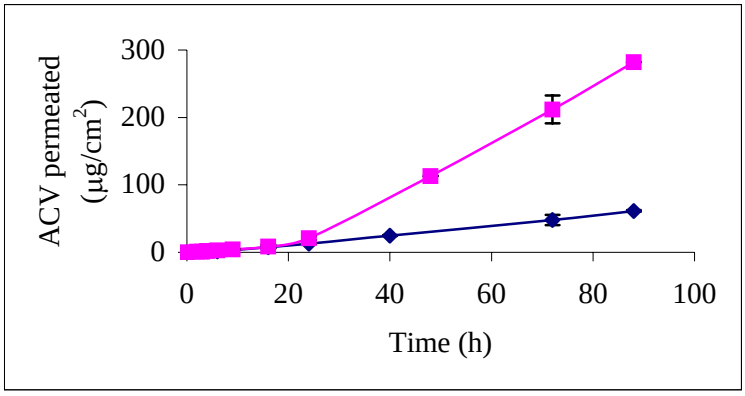


**Figure 4.** Amount of ACV permeated through porcine skin, as a function of time, from microparticles (u) and control suspension (n). Each point represents the mean ± S.E. of three experiments.